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Extraction and Fractionation of Active Protein from Microalgae *Nitzschia* sp. as Antimicrobial Agent

Sakinah Nur Fadillah[1,2], Hasnah Natsir[1*], Ahyar Ahmad[1], Abdul Karim[1], Paulina Taba[1]

[1] *Department of Chemistry, Faculty of Mathematics, and Natural Sciences, Hasanuddin University Makassar;90245, Indonesia*

[2] *Department of Medical Laboratory Technology, Sandi Karsa Polytechnic,Makassar;90245,Indonesia*

Abstract

Nitzschia sp is a marine microalgae that play a crucial role in aquaculture and can be used to enhance the nutritional value of food. *Nitzschia* sp contain a protein with the potential to be used as a novel antimicrobial agent. This study aimed to isolate and fractionate active protein from microalgae *Nitzschia sp*. and evaluate its ability to inhibit bacterial growth. *Nitzschia* sp. was cultivated, then was centrifuged to separate its filtrate from the biomass. Biomass composition contains 39.98% protein was measured by Kjeldal method. Protein was isolated from the biomass used salting-out method by adding ammonium sulfate at saturation levels of 0-20%, 20-40%, 40-60%, and 60-80%. Protein was purified by a dialysis method using a cellophane membrane. The protein fraction concentration was determined by the Lowry method. Antimicrobial activity was determined by using dis diffusion method. The results showed that the protein content of each fraction of 0-20%, 20-40%, 40-60%, and 60-80% were 10.3 mg/mL, 0.93 mg/mL, 1.61 mg/mL, and 1.91 mg/mL, respectively. The antibacterial study showed that protein fractions have an inhibitory effect on the growth of pathogenic bacteria *Salmonella thypi* (fraction 0-20%) with the largest inhibition zone of 14.3 mm and *Staphylococcus aureus* (fraction 0-20% and 60-80%) with the largest inhibition zone of 14.7 and 8.2 mm, respectively. Protein fractions from *Nitzschia* sp. were found to be effective against both gram positive *Staphylococcus aureus* and gram negative *Salmonella thypi*.

Keywords : *Nitzschia* sp. , microalgae, fractionation, antimicrobial

1. Introduction

Antibiotic resistance microorganisms have emerged over the years. Antibiotic resistence is a major public health problem in all over the world and involving the transfer of bacteria and genes between humans, animals, and environment [1,3]. Antibiotic resistance has become a serious problem and affects almost all bacterial species. Many common pathogens, such as *Staphylococci*, and *Pseudomonas* have developed resistance to multiple antibiotics.. *Staphylococcus aureus* strains are resistance to penicillin and methicillin [2], while *Salmonella thypi* strains are resistance to ampicillin [3]. One approach to overcoming drug resistance is to obtain new molecules from natural sources. To combat antibiotic resistance, microalgae have been identified as a target organism for new antimicrobial molecules. To resist exposure to bacteria pathogens, these organisms have developed tolerance and defense strategies. [2].

Microalgae are classified as phytoplankton in aquatic ecosystems [4]. Microalgae are prokaryotic or eukaryotic photosynthesis microorganisms that can grow rapidly and survive in a variety of environments [5]. Microalgae have developed complex metabolic pathways in order to survive in highly competitive environments [6]. They are especially valuable due to their high content of compounds with different biological activities such as, protein, carbohydrate, lipid, and vitamin. Microalgae have been linked to a variety of health benefits. [7] and become alternative of protein sources. Protein from microalgae has a nutrient compositions that are comparable, if not superior to conventional foods. *Nitzschia* sp. is one of the microalgae which considered as a valuable alternative protein source and one of the most abundant microalgal division [8]. *Nitzschia sp* can grow quickly, and contain 33-59% of crude protein [9,10]. Extraction and fractionation of protein from *Nitzschia* sp., as well as antimicrobial activity screening, were performed in this study.

2. Experimental

Materials used in this study were *Nitzschia* sp. Guillard, aquadest, (Tris(hydroxymethyl)amino methane 0.1 M (Merck), NaCl 2 M (Merck); $CaCl_2$ 0.01 M (Merck); β-mercaptoethanol 1% (Merck); Triton X-100 0.5% (Merck), hydrochloric acid (HCl), ammonium phosphate ($(NH_4)_2SO_4$) (Merck), Bovine

*Corresponding author e-mail: hasnahnatsir@unhas.ac.id.; (Hasnah Natsir).

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Serum Albumin (BSA) (Merck), and potassium permanganate ($KMnO_4$) (Merck), paper disc (Oxoid).

2.1. Cultivation of Nitzschia sp.

Nitzschia sp. was first cultivated in f/2 (Guillard) medium culture (designed for growing most microalgae) , aerated and incubated at room temperature and illuminated with an intensity of 2000 lux. A total of 200 ml of starter culture of *Nitzschia* sp. (pre-prepared) in a volume of 1500 mL was inoculated into the medium [9]. *Nitzschia* sp cell density was calculated from the first day (log phase) to the death phase using hemocytometer [11].

2.2. Biomass Composition

Nitzschia sp. culture was centrifugated at 10.000 rpm at 4 ^oC for 30 minutes to separate filtrate, and then dried using Freeze-drying. Ash levels were determined gravimetrically by drying at 105 ^oC for 15 minutes, then incenerating in an ashing furnace at 550 ^oC for 6 hours, and weighed until a constant weight was obtained. Measurement of protein content was carried out using the Kjeldahl micro method. Crude lipid was extracted with Soxhlet using lipid solvent. Carbohydrate content was calculated by difference (100-(moisture + ash + crude protein + crude lipid))[13].

2.3. Extraction, Fractionation, and Dialysis of Protein

Protein was extracted by cell homogeneous buffer (tris(hydroxymethyl)amino methane 0,1 M, NaCl 2 M, $CaCl_2$ 0,01 M, β-mercaptoethanol 1%, Triton X-100 0,5%) by a frozen and sonication process to obtain a crude extract of protein [14]. The fractionation of crude protein extract was then carried out at 0-20% (F1), 20-40% (F2), 40-60% (F3) and 60-80% (F4) of ammonium sulfate. Protein fractions were then dialyzed in cellophane membrane [15].

2.4. Determination of Protein Content

The determination of protein content was conducted using the Lowry Method with Bovine Serum Albumin (BSA) as a standard solution and distilled water as a blank [16].

2.5. Antimcrobial Screening

Salmonella thypi and *Staphylococcus aureus* were both tested for their resistance to protein fractions. The antimicrobial activities were conducted by using disk diffusion method. The paper disks were dipped in each protein fraction for 15 minutes and then placed on the surface of the media, incubated at 37 ^oC for 24 hours and, then the inhibition zone was observed by looking at the clear area around the paper disk [13].

3. Results and Discussion

3.1. Cultivation of Nitzschia sp.

Cultivation of *Nitzschia* sp. was carried out for 12 days. During this time, temperature, salinity, and light were controlled for optimum *Nitzschia* sp. growth. Temperature plays a role in metabolic processes, while salinity maintains the osmotic pressure, and light influences photosynthesis [17,10]. The presence of cell growth in *Nitzschia* sp. culture was indicated by the darkening of the culture. Everyday, 1 mL of *Nitzschia* sp. culture was taken to calculate cell density. The calculation of cell density was stopped on the twelfth day, when the cell density decreased. The growth curve had characteristic shape observed for microorgnisms beginning with an exponential phase.

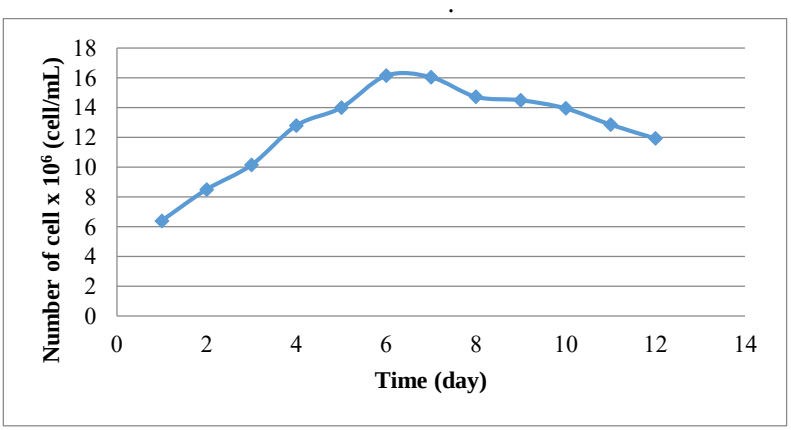

Fig. 1. *Nitzschia* sp. growth curve. The 1-5th day is log phase, the 6-7th day is the stationary phase, the 8th day is the death phase.

Nitzschia sp. entered logarithmic phase (log phase) from the first to fifth day of the experiment (Fig. 1) which is characterized by a logarithmic or exponential increase in the number of cells. The stationary phase occurred from sixth to the seventh day, where there was no increase in the number of microalgae populations. The phase of death that occurred from the eight to twelfth day, during which the microalgae population was decreased. Previous research indicates that *Nitzschia* sp. experiences the lag phase on day 1, the stationary phase on day 6 and the death phase on day 8 [17].

3.2. Biomass Compositon

The biomass composition of *Nitzschia* sp. was analyzed including ash, crude protein, crude lipid, and carbohydrate content. Table 1 shows the result of the biochemical composition of *Nitzschia* sp. Biomass of *Nitzschia* sp. contained 39.98%. crude protein. The protein content of *Nitzschia* sp. was comparable with the literature data [9] but not with the literature data [10] which showed protein content of *Nitzschia* sp. is 59.28%. Carbohydrate were present in smaller quantities. *Nitzschia* sp. biomass contained 4.99%. carbohydrate The carbohydrate and lipid content were not comparable with the literature data [10] which showed that the carbohydrate and lipid content of *Nitzschia* sp. is 31.56% and 7.76% respectively. *Nitzschia* sp. has a 22.61% ash content. The ash content of *Nitzschia* sp. not much different with the literature data [18] which showed ash content of *Nitzschia* sp . is 27.8% When compared to other types of microalgae such as *Spirulina* and *Tetraselmis chuii* with 8-16% of ash content, *Nitzschia* sp contained high level of ash or mineral

Table 1. Biomass Composition

No.	Biomass Composition	%
1	Ash	22.61
2	Lipid	32.42
3	Protein	39.98
4	Carbohydrate	4.99

3.3. Extraction, fractionation, and Dialysis of Protein

Extraction of bioactive protein from microalga using the previous research procedure *Nitzschia* sp cells were dissolved in 60 mL of buffer solution, and frozen/thawed. Upon cell lysis, sonication was carried out for 30 minutes at 4ºC. The cell lysate was then centrifuged to obtain the crude protein extract. The crude extract of protein was fractionated ammonium sulfate saturation levels of 0-20% (F1), 20-40% (F2), 40-60% (F3), and 60-80% (F4) The addition of ammonium sulfate at varying concentrations results in different types of precipitated protein at each level of the fraction. Purification process followed by dialysis with a cellophane membrane.

Dialysis was conducted to eliminate the salt that remained in the protein fraction. Buffer solution used in the outer membrane has a lower concentration than the concentration inside the cellophane membrane. The protein content was determined by the Lowry method. Figure 2 depicts the concentration of protein frcations at each level of the precipitate obtained by ammonium sulfate saturation. The Protein content of each fraction differed, indicating that the precipitated protein from each fraction was different. The highest concentration of protein fraction was found in fraction F1 and the lowest protein concentration was found in F2.

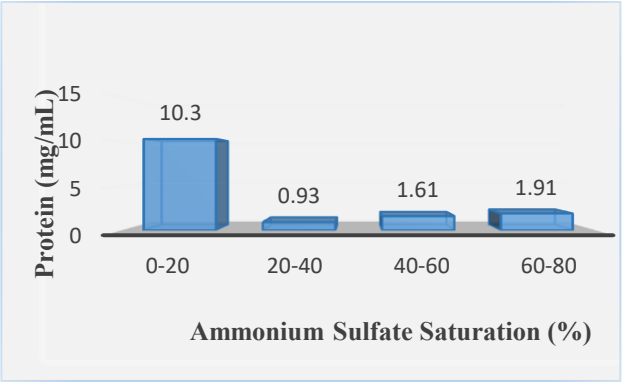

Fig. 2. Effect of the level of saturation $(NH_4)SO_2$ of the protein concentration from *Nitzschia* sp. Concentration of protein was different in each fraction.

3.4. Antimicrobial Screening

Many research studies have found increased drug resistance in human pathogens. Therefore, it is important to conduct a search for new antimicrobial agent from a new source. This research used microalgae as a new source of protein. The antimicrobial activity of protein fraction from *Nitzschia* sp. has not been extensively studied so far. The only report concerns a bioactive peptide from *Nitzschia* sp. that has been shown to be effective against bacteria gram negative *E. coli* [19]. *Nitzschia laevis*, another type of *Nitzschia*, has been reported to have antioxidant, but no antimicrobial activity against the tested organism *Sthapylococcus* [20].

In our experiment, antimicrobial activity was tested using the disk diffusion method against gram positive *Staphylococcus aureus* and gram negative *Salmonella thypi*. The purpose of using this microbe is to determine if *Nizschia* sp has a broad spectrum of antimicrobial activity. Because chloramphenicol is a bacteriostatic antibiotic with a broad spectrum, it was used as a positive control (21). As shown in Table 2, The results show that protein fractions have an inhibitory effect.

F1, F4, and chloramphenicol all had an inhibitory effect on the growth of *Staphylococcus aureus*. F1 has a 14.7 mm inhibition zone, F4 has a 8.2 mm inhibition zone and positive control has a 9.7 mm inhibition zone. Because the F1 inhibition zone was larger than the positive control , *Staphylococcus aureus* may have developed resistance to chloramphenicol.

Salmonella thypi growth inhibition was observed in F1 and chloramphenicol. F1 has a 14.3 mm inhibition zone and chloramphenicol has a 18 mm inhibition zone. According to Zone of Inhibition (ZOI) classification, the protein fraction F1 demonstrated strong antimicrobial activity, while F4 showed medium antimicrobial activity. Zone inhibition is classified based on ZOI diameters : >20 mm, very strong; 10-20 mm, strong; 5-10 mm, medium [22]. F2 and F3 had no antimicrobial activity against *Staphylococcus aureus* and *Salmonella thypi* in this study. Figure 3 depicts the diameter of protein fraction from the microalgae *Nitzschia* sp.

Table 2.Antimicrobial Activity of Protein Fractions from *Nitzschia* sp.

Protein Fraction	*Inhibition Zone (mm)*	
	Staphylococcus aureus	***Salmonella thypi***
0-20% (F1)	14.7	14.3
20-40% (F2)	0	0
40-60% (F3)	0	0
60-80% (F4)	8.2	0
Chloramphenicol (+)	9.7	18
Control negative	0	0

The size of the inhibition area is influenced by the growth rate of microorganism , the ability and rate of diffusion of the active ingredient in the medium, the sensitivity of microorganism to the active substances and the thickness of the medium's viscosity [23]. Based on the result, protein fractions from *Nitzschia* sp. were effective against both gram positive and gram negative bacterial strain and have a board spectrum.

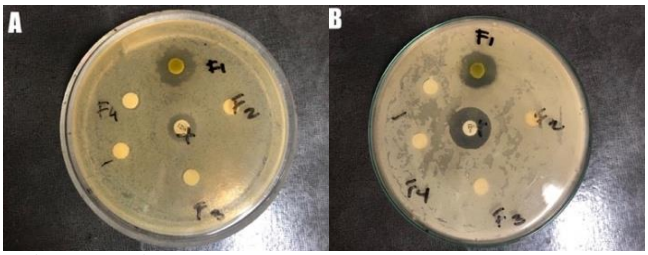

Fig. 3. Visualization of Diameter of Protein Fraction against *Staphylococcus aureus* (a) and *Salmonella thypi* (b)

4. Conclusions

Protein exploration reveals antimicrobial activity of microalgae, especially *Nitzschia* sp. The result showed that protein fractions of *Nitzschia* sp. inhibit the growth of pathogenic bacteria, with fractions 0-20% (F1) inhibiting the growth of *Salmonella thypi* (inhibition zone of 14.3 mm) and fractions 0-20% (F1) and 60-80% (F4) inhibiting the growth of *Staphylococcus aureus* (inhibition zone of 14.7 and

8.2 mm, respectively). Further studies are required to investigate the antimicrobial activity of protein fraction againts fungi and yeast.

5. Conflicts of interest

The authors declare that there is no conflict of interest in the research results or publication**.**

6. Acknowledgments

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